Genetic and Phenotypic Characterization of *Listeria monocytogenes* Strains with Attenuated Virulence

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Abstract

Epidemiological studies have provided considerable evidence that *Listeria monocytogenes* strains differ in their ability and relative likelihood to cause human disease. Multiple mutations leading to premature stop codons (PMSCs) have been identified in the key *L. monocytogenes* virulence gene *inlA,* which codes for a surface protein that mediates the entry of *L. monocytogenes* into host cells. Mutations leading to premature stop codons in *inlA* are associated with a reduced invasion phenotype *in vitro,* supporting the hypothesis that these mutations may also be responsible for attenuated human virulence. In order to further define the frequency of *L. monocytogenes* strains with reduced invasion capability, 207 isolates had previously been screened for ability to invade Caco-2 cells (Nightingale, unpublished). The *inlA* gene was sequenced for 27 isolates that showed attenuated ability to invade the epithelial cell line. Sequencing revealed mutations leading to PMSCs in 12 of the 27 isolates, including 2 novel mutations (PMSC types 5 and 7) and 3 previously reported mutations (PMSC types 1, 4 and 6). All but one of the isolates with confirmed PMSC mutations in *inlA* were isolated from food products or food processing plants. Our data support the hypothesis that *L. monocytogenes* isolates associated with food have attenuated invasion due to the presence of *inlA* PMSCs. Our data further suggest that sequence variation in *inlA* is an important factor that can be used to define *L. monocytogenes* that have a reduced ability to cause human disease. Since some strains with PMSCs in *inlA* were common in foods and

environmental samples and were found in high levels in foods, it was hypothesized that these mutations may provide selective advantage, possibly outside a human host. Isogenic *inlA* PMSC strains were thus tested for their growth in rich medium and their resistance against common sanitizing agents. The results indicate that there is little difference in growth trends or sanitizer resistance between isogenic strains with and without *inlA* PMSC. It thus appears that *inlA* PMSCs do not enhance the ability of *L. monocytogenes* to grow in rich media or to be resistant to sanitizing agents.

Introduction

Listeria monocytogenes is a gram-positive, facultative intracellular bacterium responsible for foodborne listeriosis, a severe disease which is responsible for approximately 500 deaths in the US every year, particularly in people who are immunocompromised (Mead et al., 1999). *L. monocytogenes* is able to cross the blood-brain barrier resulting in meningitis and encephalitis, and can also cross the feto-placental border to cause spontaneous abortions in pregnant women. Because *L. monocytogenes* is transmitted through food, the food industry has made attempts to control the transmission of this pathogen. Recalls of Ready-to-Eat (RTE) foods are often instituted due to the presence of *L. monocytogenes*. Because this bacterium is common in many environments, *L. monocytogenes* contamination of foods has been costly to the food industry and targeted interventions are needed to reduce the presence of this pathogen in the food chain. Recent epidemiological studies have provided evidence that *L. monocytogenes* strains differ in their ability and relative likelihood to cause disease (Gray et al., 2004; Nightingale et al., 2005a). Only three of thirteen known serotypes of *L.*

monocytogenes (4b, 1/2a and 1/2b) are responsible for 90% of human cases (McLauchlin, 1990).

L. monocytogenes initiates infection in humans by invading intestinal epithelial cells. Internalin A (InlA) is a bacterial surface protein encoded by *inlA*. Through its interactions with the host receptor, E-cadherin, InlA allows the bacteria to invade human intestinal epithelial cells (Lecuit, 2001). Multiple mutations leading to premature stop codons have been identified in *inlA* (Nightingale et al., 2005; Olier et al., 2002 and 2003; Jonquieres et al., 1998; Rousseaux et al., 2004). All of the known mutations lead to production of a shortened form of the InlA protein which is missing its LPXTG motif, the sequence necessary to anchor the surface protein to the peptidoglycan in the bacterial cell wall (Dhar et al., 2000). This truncated form is no longer attached to the surface, but instead is secreted (Jonquieres, 1998). Mutations leading to premature stop codons in *inlA* are associated with a reduced invasion phenotype *in vitro*, supporting the hypothesis that these mutations may also be responsible for attenuated human virulence (Nightingale et al., 2005a).

As isolates with premature stop codons in *inlA* are prevalent in food and environmental samples and often grow to high levels in foods (Nightingale et al., 2005a), it was hypothesized that this mutation must provide evolutionary advantage, most likely in a non-host environment. Since many of the *inlA* PMSC mutations have been found in isolates from food sources while very few mutations have been found in isolates from human cases, it seems possible that the mutation may confer some sort of advantage for survival in the food processing environment. Since sanitizing agents are commonly used in these environments, it was hypothesized that the *inlA* PMSCs may provide sanitizer

resistance. Different strains of *L. monocytogenes* have been shown to have differing sanitizer resistance (Romanova et al., 2002), however, nobody has tested whether this resistance correlates with *inlA* PMSCs.

Previous studies (Nightingale et al., 2005a; Olier et al., 2002; Jonquieres et al., 1998; Rousseaux et al., 2004; Orsi, unpublished data) have reported 10 distinct mutations leading to *inlA* premature stop codons (Table 1). Additionally, it has been shown that *L. monocytogenes* mutants with *inlA* PMSCs show attenuated virulence as compared to wild type (WT) strains (Nightingale et al., 2005a). The purpose of this study was to (i) confirm that an association exists between invasion-attenuated isolates and *inlA* non-sense mutations; (ii) define the prevalence of *Listeria monocytogenes* isolates with *inlA* PMSCs among various ribotypes, and (iii) determine whether any of these mutations confer a selective advantage outside the human host.

Materials and Methods

Bacterial Strains and Growth Conditions

L. monocytogenes isolates (n=207) were chosen from our collection of isolates, consisting of human, food and environmental isolates collected from across the United States (Supplementary Table 1). Isolates were chosen to represent ribotypes (n=88) described in Gray et al., (2004) and Lappi et al., (2004) for which invasion efficiency was not previously established. Between 1 and 4 isolates were tested for each ribotype. Isolates were chosen based on ribotype representation in our isolate collection. Specifically, if less than 4 isolates were available for a given ribotype, only 1 was chosen for testing, whereas if >20 isolates were available, 4 were tested. If between 4 and 20

isolates with a given ribotype were found in our collection, 2 or 3 isolates were chosen. Isolates represented human, food and environmental sources (Supplementary Table 1). *inlA* sequence information on these isolates had not been previously reported.

Isogenic Pairs Description

Growth of two sets of isogenic pairs was evaluated for sanitizer resistance and growth rate. Mutants used were generated previously by site directed mutagenesis, and these previously generated mutants were acquired for this study (Nightingale et al., 2005a). Each set consisted of two isogenic pairs (Table 3). The first pair in each set consisted of a wild type isolate and the same isolate with an introduced *inlA* PMSC. The second pair in each set consisted of an isolate with a naturally occurring *inlA* PMSC and the same isolate in which the PMSC was reverted back to "wild-type," encoding full length InlA. The first set evaluated PMSC 3, while the second set evaluated PMSC 1.

Caco-2 Invasion Assay

For infections, *L. moncytogenes* cultures were grown overnight at 30°C in brain heart infusion broth (BHI) at a 45° angle without shaking. The cultures were centrifuged (5 min @ 13,000 RPM) and re-suspended in 1 ml of phosphate buffered saline (PBS) before they were used for invasion assays.

Caco-2 human intestinal epithelial cells (ATCC HTB-37) were cultured in Dulbecco's minimal essential medium (DMEM) containing 20% fetal bovine serum and penicillin/ streptomycin. The cells were grown at 37°C under a 5% CO_2 atmosphere with a relative humidity of 80 to 95%. For the invasion assay, the Caco-2 cells were seeded in

a 24-well plate and grown for 48 hours as described above, except that the media used did not contain antibiotics.

Invasion assays were performed as previously described (Nightingale et al., 2005a). Briefly, confluent Caco-2 cell monolayers in a 24-well plate were inoculated with 10.5 µl of undiluted *Listeria* cell suspension in PBS (approximately equivalent to 2 x 10^7 bacteria/well). Inoculated Caco-2 cells were incubated for 30 minutes at 37°C, followed by three PBS washes. The cells were then incubated with fresh media. At 45 minutes post-infection, media with 150 µg/ml gentamicin was added. At 90 minutes post-infection, the cells were washed 3 more times with PBS. The Caco-2 cells were then lysed by addition of ice-cold water with vigorous pipetting to lyse the cells. Bacterial cells were enumerated on BHI agar plates by Spiral Plater (Spiral Plater, Biotech). Invasion efficiency was reported as percentage of intracellular bacteria recovered as compared to the amount recovered from the control strain of *L. monocytogenes*. 10403s was thus used as a positive invasion control and a Δ*inlA* strain was used as a negative invasion control. Additionally, an uninoculated BHI well was used as a negative control for the assay.

inlA Sequencing

Isolates that showed consistently low invasion ability were chosen for DNA sequencing of *inlA* (n=27). PCR was performed using *Taq* polymerase. PCR products were purified, quantified and sequenced as described previously (Nightingale et al., 2004). *inlA* was sequenced as described in Table 2. The 3' end was sequenced first, followed by additional 800-bp sections in the 5' direction until a premature stop codon

(PMSC) was found or until the entire open reading frame (ORF) was sequenced. The 3' end was sequenced first because all previous *inlA* PMSCs identified in our lab were in the 3' end of the gene (Nightingale, 2005a). Additionally, the promoter region was sequenced in 4 of the isolates in which PMSCs were not found in the ORF. The *inlA* sequences were all compiled and aligned using MegAlign (Lasergene software suite, DNAStar, Madison, WI). The correct reading frame was established by using the previously determined start codon for *inlA* (Glaser et al., 2001). In isolates where no PMSC was found, invasion assays were repeated in duplicate to reconfirm attenuated invasion.

Hemolysin Activity Detection

Isolates with reduced invasion (n=26) were streaked on blood agar plates to test for hemolysin activity. One human isolate (FSL M2-036) with a detected PMSC was not tested because its attenuated invasion was already shown to be attributed to *inlA* mutations (this study). The plates were incubated at 37°C overnight before they were inspected visually for clearing around bacterial colonies, indicating hemolysis.

Growth Curve

Isolates with premature stop codons in *inlA* have shown decreased virulence but are prevalent among environmental and food isolates, indicating that this mutation might provide an evolutionary advantage to the bacteria. Growth rates in a rich medium of brain heart infusion broth (BHI) were compared in isogenic *inlA* mutant strains to determine whether *inlA* PMSC may allow isolates to grow faster.

Isogenic pairs were evaluated, as described above. Strains were grown overnight in BHI at 37°C with shaking. From the overnight culture, 50 µl was transferred to 5 mL of fresh BHI and incubated at 37 °C with shaking. When cultures reached an O.D.600=0.4, 500 µL was transferred to 50 mL BHI in a side arm flask. Flasks were incubated at 37°C with shaking. Optical density readings were taken every hour for 12 hours. Cell counts were determined at 0, 4, 8, and 12 hours by Spiral Plater (Spiral Plater, Biotech).

Sanitizer Minimum Inhibitory Concentration (MIC)

Sanitizer resistance was tested for isolates with *inlA* PMSCs versus WT isolates. The two isogenic mutant sets as described above (table 3) were also used in these experiments.

Bacterial cultures were incubated at 37° C without shaking for 18 hours. Two ten fold dilutions were performed on the cultures to get a final cell count of about 1 x 10^7 CFU/ml.

Both a phosphoric acid (Dividend Anionic Acid Sanitizer; manufacturer's suggested concentration: 4 µl/ml) and an ammonium based sanitizer (Render II Quarternary Disinfectant; manufacturer's suggested concentration: 16 µl/ml) were tested in this study. Sanitizers were prepared at four times the manufacturer's suggested concentration and serially diluted (2-fold) into PBS across a row of a 96 well plate. 2X BHI was then added to each well. Finally, 5 µl of inoculum was added to each well.

Seven isolates can be tested on one plate along with a negative control. The last column on each plate was reserved as a negative column in which no bacteria was added.

Plates were incubated for 24 hours at 37° C before OD readings were taken on the Fusion spectrophotometer at 600 nm. MIC was defined as the lowest concentration of sanitizer in which no *L. monocytogenes* grew.

Results and Discussion

Distinct *inlA* PMSCs were Found in Isolates Showing Low Invasion Capacity including 2 Novel Mutations

Since ability to invade human epithelial cells in vivo is a crucial step in the virulence of *L. monocytogenes* (Lecuit, 2001), we hypothesized that the inability to invade Caco-2 human epithelial cells in vitro may indicate reduced human virulence. In initial tissue culture experiments, 207 isolates were screened for invasion efficiency in Caco-2 cells by Dr. Kendra Nightingale (Nightingale, unpublished). From this initial screen, isolates with decreased ability to invade Caco-2 cells (n=27), which was defined as invasion efficiency <25% in each of two independent trials, were chosen for *inlA* sequencing. I screened these 27 isolates for *inlA* PMSCs which have been previously associated with attenuated invasion in Caco-2 (Nightingale, not published). The screen was done beginning with the 3' end because this is where PMSCs were previously identified, however, the entire ORF was sequenced for all of the isolates, except for two in which PMSCs were found in the initial 3' end screen. Five distinct PMSCs were found across 12 of these 27 isolates with attenuated invasion (Tables 1 and 2). While three of these mutations were previously described (i.e. PMSC # 1, 4 and 6), two of these are being described in this study for the first time (i.e. PMSC # 5 and 7). In isolates

where no PMSC was found, invasion assays were repeated in duplicate to reconfirm attenuated invasion. Of the 15 isolates that were re-screened for invasion ability, only one (FSL M2-035) was confirmed to have attenuated invasion (Table 2). The sequence will have to be screened for other non-synonomous mutations that are not necessarily nonsense mutations. It was also hypothesized that the attenuated invasion in this isolate may be due to mutations in upstream regulators of *inlA*. Since PrfA activity was shown to be normal in all isolates tested (as indicated by hemolysin results, below), it is possible that the attenuated invasion in this isolate is due to a *sigB* mutation. Sigma B dependent activation has been found to be important for *inlA*-mediated entry into epithelial cells (Garner et al., 2006).

To date, 12 different *inlA* PMSCs have been reported (Table 1). A previous study has shown that mutation types 1-3 result in a truncated, secreted form of the *inlA* protein as opposed to the cell surface protein form which aids in invasion (Nightingale et al., 2005a). While no western blot was done for the 2 newly reported mutations, both mutations occur before the LPXTG motif, which is crucial in anchoring the protein to the bacterial cell wall (Dhar et al., 2000), and thus, we hypothesized that any isolate with either mutation would not be able to produce a form of the protein that is anchored to the cell wall. The newly reported mutations (PMSC 5 and PMSC 7) are both substitutions of a cytosine for a thymine, resulting in a premature stop codon (Table 1 and Supplemental Table 2).

Attenuated Invasion due to *inlA* PMSCs Was Widespread Across Ribotypes

While previous studies have focused on predominantly food-associated ribotypes (Gray et al., 2004; Nightingale et al., 2005a), this study screened a more comprehensive set of isolates from 88 different ribotypes for invasion ability in Caco-2 cells. The isolates were from a variety of sources, including, food and food processing plants, farm environments and human clinical cases. Of the 88 ribotypes screened for invasion ability, isolates from 19 ribotypes (ie. the 27 isolates described in the above section) were chosen for *inlA* sequencing based on initial invasion assay results. Among these 19 ribotypes, 8 ribotypes were shown to contain isolates with mutations (Table 4). None of these ribotypes had previously been screened for invasion efficiency.

Interestingly, all of the *inlA* PMSCs were found in lineage II isolates, with the exception of one human isolate (FSL M2-036). Lineage II isolates are more commonly found in food and environmental sources than in human clinical cases (Nightingale et al., 2006) and our data further support that the underrepresentation of lineage II isolates in human listeriosis cases may be partially due to a high frequency of strains with *inlA* PMSCs in this lineage. In the four cases where more than one isolate was tested for a given ribotype, all of the isolates in that ribotype were shown to contain a PMSC. The two ribotype DUP-1045b isolates tested each contained a different PMSC, while a previous study has shown that each ribotype only has one PMSC associated with it (Nightingale et al., 2006). The diversity of these mutations in this ribotype may be due to the fact that these isolates are generally associated with environmental sources, making them less likely to retain virulence factors.

Hemolysin Activity was Present in All Strains with Attenuated Invasion

While attenuated invasion in *L. monocytogenes* has often been associated with mutations in *inlA*, it has been hypothesized that mutation in upstream regulators of the gene may also be involved in some cases. *prfA* is an important upstream regulator of many virulence factors, including *inlA* and hemolysin (*hly*), a gene involved in lysing host cells (Geoffroy et al., 1987). Since *hly* expression is fully dependent on PrfA, positive hemolysin activity is a good indication of PrfA activity.

Since one isolate was found to have consistently low invasion without containing a PMSC, it was hypothesized that this attenuated invasion may be due to reduced PrfA activity. However, this isolate, along with the other 25 isolates with attenuated invasion that were tested for lysis activity on red blood cells, were all shown to have positive hemolysin activity (data not shown). *inlA* has been shown to be controlled by PrfA-dependent and independent mechanisms (Lingnau et al., 1995). Thus, it is possible that a mutation in another upstream regulator, such as *sigB* (Kim et al., 2005) is responsible for the attenuated invasion in FSL M2-035.

***inlA* Mutations Did Not Confer any Growth Advantages in Rich Medium**

Isogenic *inlA* mutant pairs were tested for growth in BHI at 37°C in a single trial. In all pairs except one, the results indicate that there is little difference in growth between a wild type (WT) *L. monocytogenes* isolate and the same isolate with an *inlA* PMSC (Figures 1 and 2). In pair 2-1, however, the mutant N4-730 in which a naturally occurring PMSC was reverted back to a WT full length *inlA* allele showed decreased growth compared to the same strain with the naturally occurring PMSC 3 (Figure 2).

However, the cell counts for N4-730 were actually higher than its isogenic strain (F2-515) at 8 and 12 hours, indicating that the lower absorbance readings may be due to smaller size of N4-730. Additionally, the isolates in the other pair in set 1, which shows PMSC 3 introduced into a wild type strain, showed no difference in growth trends between them. Therefore, we conclude that it is most likely that during construction of strain FSL N4-730, additional genetic changes occurred that affected the cell size or OD readings of this strain.

inlA Mutations Did Not Confer Resistance Against Phosphoric Acid and Ammonium Sanitizers

Since *inlA* PMSCs have been overrepresented among food isolates (Nightingale et al., 2006), it seems possible that the mutation may give the bacteria an advantage in the food-processing environment. A previous study showed sanitizer resistance to be important in bacterial survival and growth in hospital settings (Sagripanti et al., 1997). Since sanitizers are used widely in food processing facilities, mutations that confer sanitizer resistance may be equally important in these environments. Additionally, it has been shown that sanitizer resistance in *L. monocytogenes* is linked to the presence of plasmid DNA (Romanova et al., 2002), indicating that it may be a commonly acquired property. The minimum inhibitory concentration (MIC), the lowest concentration of a given sanitizer at which the bacteria can no longer grow, was determined for each of the 8 isolates as single trials (Table 5). There were no differences in sanitizer resistance between a WT isolate and a mutant isolate in any given pair. This held true for both types of sanitizer- phosphoric acid and ammonium. The only differences in resistance

were between strains, which could be due to any number of factors. For instance, 10403s and its isogenic mutant, W3084, were both more resistant to the phosphoric acid sanitizer and the ammonium sanitizer than any of the other strains tested. While this may be an interesting avenue for further study, it was beyond the scope of the present study. Based on the MICs, *inlA* PMSCs do not affect resistance against phosphoric acid or ammonium sanitizers.

Conclusions

This present study, along with other studies that have identified *inlA* PMSCs (Nightingale et al., 2005a; Olier et al., 2002 and 2003; Jonquieres et al. 1998; Rousseaux, 2004), has demonstrated that nonsense mutations in *inlA* can be strong indicators of invasion ability, and, therefore, theoretically its ability to cause disease in humans. Since the food industry's fight against *L. monocytogenes* can be very expensive, it would be extremely valuable to be able to determine which strains are the most dangerous. The food industry could then focus its efforts on these strains.

While *inlA* PMSCs have been predominantly found in food and environmental isolates, there have been PMSCs identified in isolates from human clinical cases (this paper; Nightingale et al., 2005a). This may indicate that the *inlA* mutation is not a definitive method for determining virulence in *L. monocytogenes*. However, the full background information on the human isolate is not known, meaning the patient could have been immunocompromised. In a previous study, a patient with a compromised immune system due to lymphoma was shown to be infected with a *L. monocytogenes*

strain that was not found to cause any further disease in other humans (Fioriti et al., 2006), providing evidence that an otherwise non-pathogenic strain can cause disease in certain patients. In this case, it was not known whether the strain carried an *inlA* PMSC or not. Additionally, a study has shown that *L. monocytogenes* strains carried asymptomatically by humans have a high incidence of *inlA* PMSCs, and these strains are unable to cause infections in the human host (Olier et al., 2003). The fact that these mutations tend to be strongly correlated with food instead of clinical cases is very compelling evidence that isolates containing these mutations can not cause disease in humans. Additionally, the fact that the isolates containing *inlA* PMSCs are severely invasion attenuated is further evidence for attenuated virulence in these isolates, since invasion of epithelial cells is a crucial step in the virulence pathway of the bacteria.

One crucial finding of this study was the fact that *inlA* PMSCs are so widespread and prevalent in a variety of sources. Of the 19 ribotypes screened for PMSCs, mutations were found in 8. Now that distinct mutations have been identified that lead to attenuated invasion, new assays can be developed to rapidly test for the presence of these mutations. A Multiplex PCR assay has already developed to determine serotypes in *L. monocytogenes* (Doumith et al., 2004), which can theoretically be extended to test for *inlA* mutations. Additionally, a PCR-RFLP test has been developed that can rapidly screen for *L. monocytogenes* isolates with attenuated invasion in Caco-2 cells due to *inlA* PMSCs (Rousseaux et al., 2004). While this PCR-RFLP test shows promise, the authors indicate that more information on *inlA* point mutations must be accumulated to make the test more comprehensive and to allow it to detect different *inlA* PMSC mutations. The sequencing information from this study will contribute to that necessary information.

The fact that *inlA* mutations are prevalent across a variety of ribotypes and sources indicates that this mutation provides a selective advantage. Given the obvious virulence disadvantage that this mutation confers to the bacteria, it seems that this mutation would have been selected against over time to the point of extinction. Instead it has become prevalent across many different ribotypes. There is likely some advantage conferred by the PMSCs which allow the mutation to propagate. In this present study, two important phenotypic traits unrelated to virulence were studied in an attempt to identify selective advantages that may be associated with *inlA* PMSCs. First, the growth rate in rich medium was found to be the same between isolates with and without an *inlA* PMSC. However, given the fact that *L. monocytogenes* must often survive harsh conditions such as refrigeration to persist in food, it would be logical to expand this study to test growth under additional stress conditions. Secondly, resistance to ammonium and phosphoric acid sanitizers was found to be unrelated to *inlA* PMSCs. This study will need to be expanded to test other sanitizers and antibiotics commonly used against *L. monocytogenes* to further confirm that enhanced resistance to antimicrobials is not associated with *inlA* PMSCs.

This is the first study to examine the association between these phenotypic traits and the presence of *inlA* PMSCs. While no correlations were found between the mutations and the traits examined, the fact that these mutations were found to be prevalent across many sources and ribotypes indicates that the *inlA* PMSCs do confer some sort of evolutionary advantage. It would, therefore, be worthwhile to study the relationship between *inlA* PMSCs and other phenotypic traits important in the survival and growth of *L. monocytogenes*.

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Tables

Table 1:
All Observed PMSCs

PMSC	Nucleotide # and Mutation*	Mutation Type	Length of Predicted Protein	Reference	Example Isolate w/ PMSC
1	1818 T→A	Substitution	605 aa	Nightingale et. al., 2005	FSL F2-275
2	1966 C→T	Substitution	655 aa	Nightingale et. al., 2005	FSL R2-074
3	2100 C→G	Substitution	699 aa	Nightingale et. al., 2005	FSL R2-275
4	12 Deletion of A	Deletion	8 aa	Unpublished, R. Orsi	FSL R2-115
5	565 C→T	Substitution	188 aa	This study	FSL R2-080
6	1474 C→T	Substitution	491 aa	Olier et. al., 2002	FSL R2-167
7	1684 C→T	Substitution	561 aa	This study	FSL L3-401
8	1637 Deletion of A	Deletion	576 aa	Jonquieres et. al, 1998	FSL F7-001
9	1380 G→A	Substitution	459 aa	Rousseaux et. al., 2004[t]	NV8
10	1540 Deletion of G	Deletion	518 aa	Rousseaux et. al., 2004[t]	NV7
11	1961 Insertion of T	Insertion	676 aa	Rousseaux et. al., 2004[t]	NV4
12	2054 G→A	Substitution	684 aa	Rousseaux et. al., 2004[t]	NV5

* Relative to EGDe sequence
[t] Mutation location reported differently in reference

Table 2. All Sequenced Isolates with Invasion Data

isolate	ribotype	lineage	source	%inv Rep1	%inv Rep2	%inv Rep3	%invRep4	%inv Rep5	Stop codons present	PMSC Type	Part Sequenced[1]
fslr2080	dup1029a	2	food	0.92	9.87	5.61			1	5	B
fslr2081	dup1029a	2	food	2.05	5.69	7.37			1	5	C
fsll3401	dup1048a	2	foodproc	2.77	4.84				1	7	B
fslt1061	dup1039c	2	foodproc	7.81	12.99				1	7	B
fslr2167	dup1045b	2	food	7.68	1.12				1	6*	B
fslt1045	dup1062d	2	foodproc	0	0.31				1	6*	B
fslt1269	dup1045b	2	foodproc	0	0.14				1	4	B
fslr2493	dup1056a	2	food	0.46	0.92				1	4	B
fslr2551	dup1056a	2	food	0.48	0.08				1	4	B
fslr2200	dup1041a	2	food	4.8	1.19				1	4	B
fslr2115	dup1041a	2	food	5.99	0.55				1	4	B
fslm2036	dup1052	1	human	0.41	1.14				1	1	D
fsln3034	dup1039a	2	farm	1.23	4.42		N/A	328.60	0	N/A	A
fslt1229	dup1043a	1	foodproc	1.13			129.52	112.11	0	N/A	A
fsls4470	dup1057b	2	genenv	3.46	9.65		88.94	82.20	0	N/A	A
fsls6023	dup1042c	1	genenv	0.3	14.33		32.45	130.51	0	N/A	A
fslf2208	dup10148	3	human	17.07	58.83		18.25	135.67	0	N/A	B
fsls4032	dup1058c	2	genenv	0.01	47.77		152.97	150.89	0	N/A	B
fsls4899	dup1053c	2	genenv	1.02	83.44		130.47	138.45	0	N/A	B
fsls6106	dup1046a	2	genenv	3.74	33.86		129.08	166.84	0	N/A	B
fsls4839	dup1061a	3	genenv	4.02	68.25		121.24	263.47	0	N/A	B
fsls4848	dup1042b	1	genenv	6.57	64.24		39.39	235.78	0	N/A	B
fsls4548	dup1053b	2	genenv	7.98	45.03		94.44	54.53	0	N/A	B
fsle1258	dup1023b	2	farm	10.49	tntc	53.8	119.26	106.25	0	N/A	B
fsln3175	dup1045e	2	farm	10.96	63.58		107.88	62.65	0	N/A	B
fslm2035	dup1054a	2	human	17.01	29.89		8.44	0.70	0	N/A	B
fslf2086	dup10142	3	human	23.82	75.3		134.85	43.81	0	N/A	B

[1]Portion of inlA Sequenced (inlA was sequenced in sections 3' to 5' until a PMSC was found or until the entire ORF was sequenced):

A: Entire ORF + Promoter Region Sequenced

B: Entire ORF Only Sequenced

C: Entire ORF minus 108 Nucleotides at 5' End Sequenced

D: 3' End Only Sequenced

Table 3: Isogenic Mutant Strains Used[1]

Isogenic Pairs	Description
Set 1:	
Pair 1-	
10403s	Standard laboratory control strain
W3084	Premature stop codon type 3 introduced into 10403s background
Pair 2-	
FSL F2-515	Wildtype DUP-1062A strain with premature inlA stop codon type 3
FSL N4-730	Reverted premature stop codon to full length inlA in F2-515 background
Set 2:	
Pair 1-	
FSL F2-563	Wildtype DUP-1052A strain with premature inlA stop codon type 1
FSL N4-734	Reverted premature stop codon to full length inlA in F2-563 background
Pair 2-	
FSL F2-245	Wildtype DUP-1052A strain with inlA gene that encodes full-length inlA protein
FSL N4-733	Premature stop codon type 1 introduced into FSL F2-245 background

[1]These mutants were previously prepared by site-directed mutagenesis (Nightingale et al., 2005a)

Table 4: Ribotype Breakdown- Ribotypes in which isolates with *inlA* PMSCs were identified in this study. Source data is given for each ribotype based on isolates in our collection.

Ribotype	# human	# food/gen env	# animal	PMSC Type (# Found)
dup1062d	1	2	1	6(1)
dup1056a	0	2	0	4(2)
dup1052	4	0	0	1(1)
dup1048a	0	1	0	7(1)
dup1045b	0	3	1	4(1), 6(1)
dup1041a	0	2	0	4(2)
dup1039c	0	3	1	7(1)
dup1029a	0	2	0	5(2)

Table 5: MIC Data[1]

Isolate Pair	**MIC of Dividend Anionic Acid Sanitizer**	**MIC of Render II Quarternary Ammonium[2]**
10403s (No PMSC) and W3-084 (PMSC 3)	0.25 µl/ml (0.15 PPM phosphoric acid and 0.013 PPM dodecylbenzene sulfuric acid)	1.5×10^{-2} µl/ml(3.375×10^{-4} PPM benzyl chlorides and 3.375×10^{-4} PPM ethylbenzyl chlorides)
FSL F2-515 (PMSC 3) and FSL N4-730 (No PMSC)	1 µl/ml (0.6 PPM phosphoric acid and 0.05 PPM dodecylbenzene sulfuric acid)	3.1×10^{-2} µl/ml (6.98×10^{-4} PPM benzyl chlorides and 6.98×10^{-4} PPM ethylbenzyl chlorides)
FSL F2-563 (PMSC 1) and FSL N4-734 (No PMSC)	2 µl/ml (1.2 PPM phosphoric acid and 0.1 PPM dodecylbenzene sulfuric acid)	3.1×10^{-2} µl/ml (6.98×10^{-4} PPM benzyl chlorides and 6.98×10^{-4} PPM ethylbenzyl chlorides)
FSL F2-245 (No PMSC) and FSL N4-733 (PMSC 1)	2 µl/ml (1.2 PPM phosphoric acid and 0.1 PPM dodecylbenzene sulfuric acid)	1.5×10^{-2} µl/ml(3.375×10^{-4} PPM benzyl chlorides and 3.375×10^{-4} PPM ethylbenzyl chlorides)

[1] MICs are based on a single trial with each strain.
[2] Benzyl chlorides refer to n-Alkyl dimethyl benzyl ammonium chlorides and Ethylbenzyl chlorides refer to n-Alkyl dimethyl ethylbenzyl ammonium chlorides

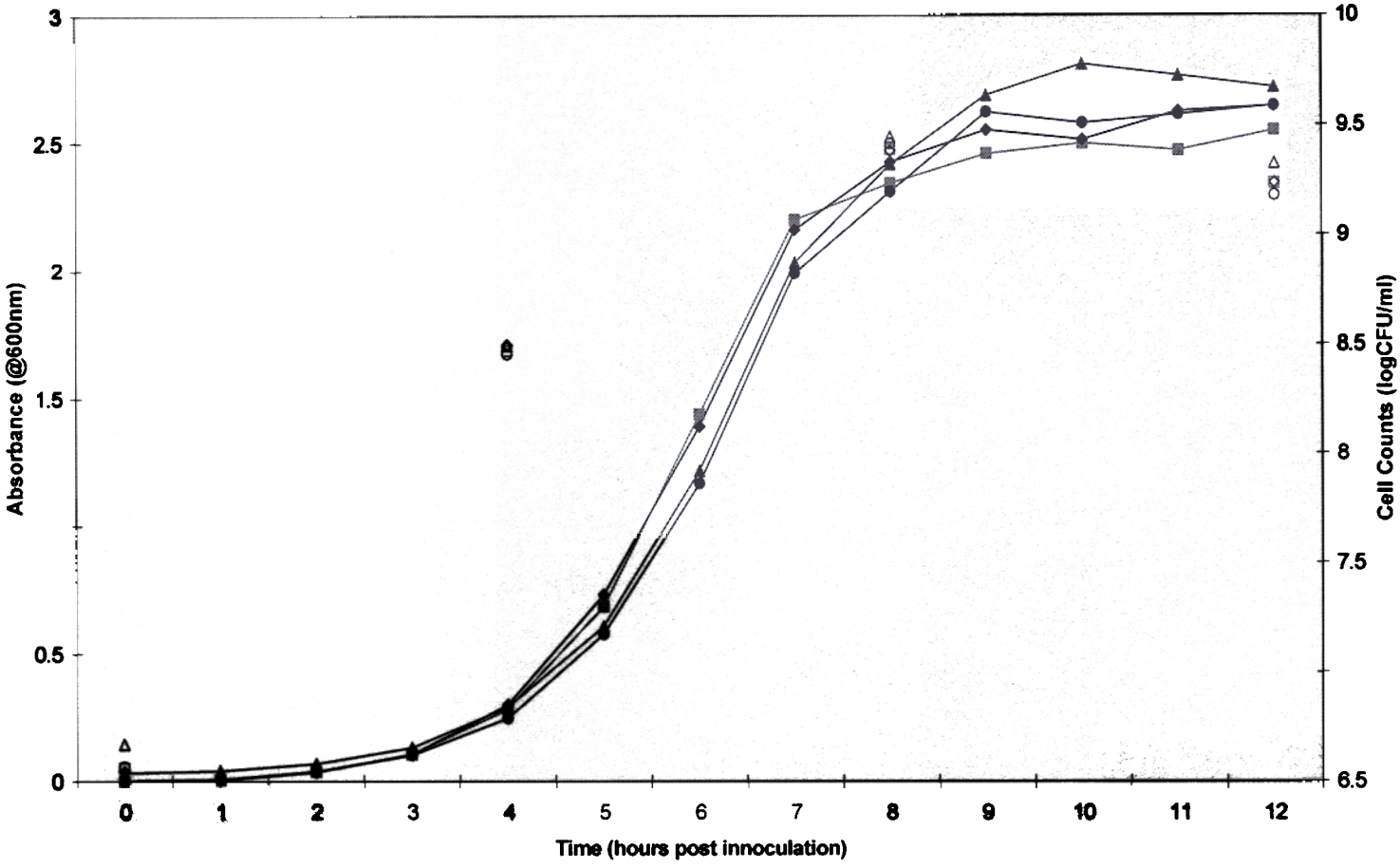

Figure 1. Growth of *Listeria monocytogenes* isogenic mutant pairs with *inlA* PMSC 1 (Set 2-Table 1). OD readings and cell counts are shown for F2-245 (□), N4-733 (♦), F2-563 (Δ) and N4-734 (O). Closed symbols represent absorbance readings and open symbols represent cell counts.

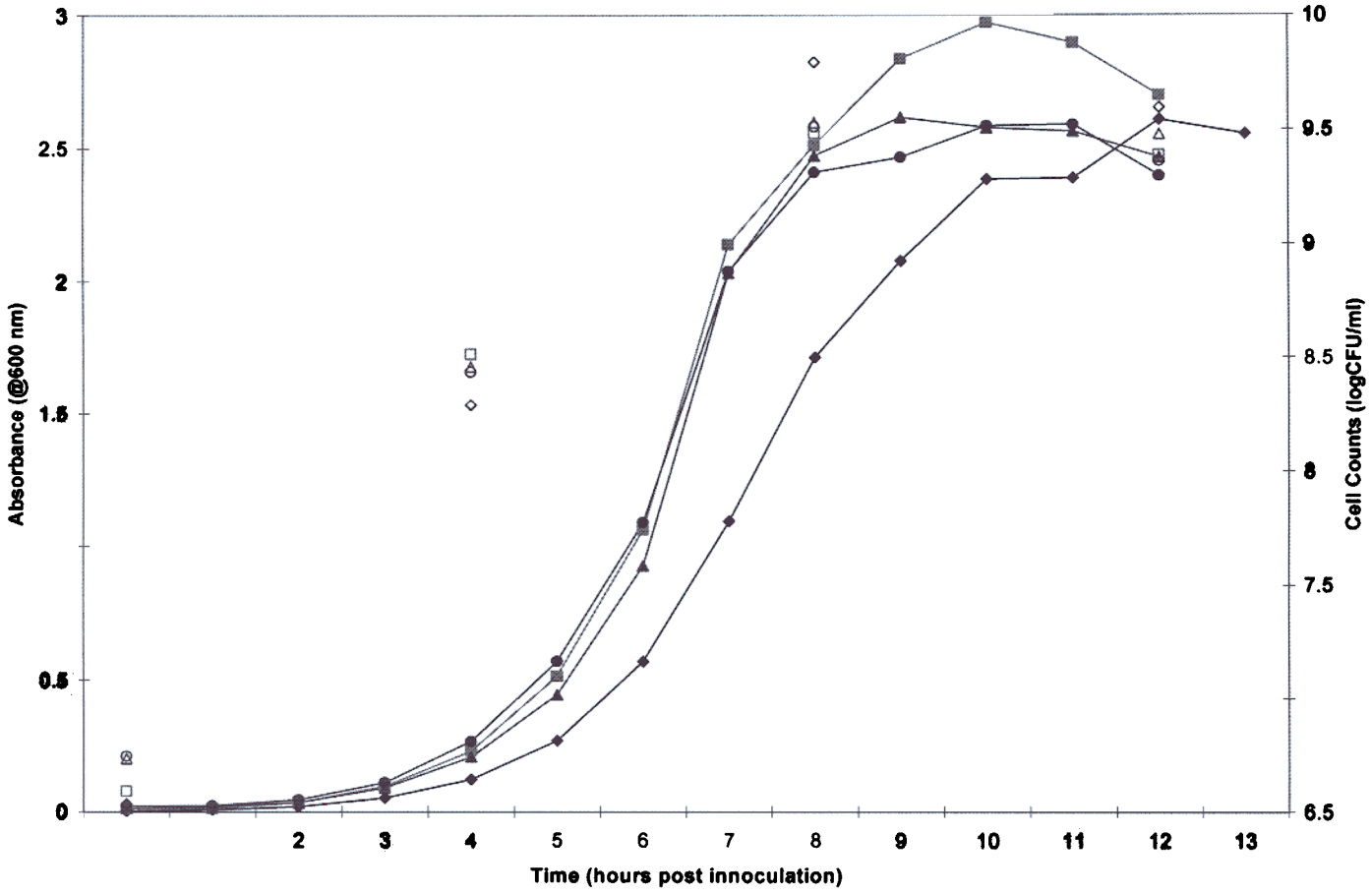

Figure 2. Growth of *Listeria monocytogenes* isogenic mutant pairs with *inlA* PMSC 3 (Set 1-Table 1). OD readings and cell counts are shown for F2-515 (□), N4-730 (♦), 10403S (Δ) and W3-084 (O). Closed symbols represent absorbance readings and open symbols represent cell counts.

Descriptions for Supplementary Tables

Supplementary Table 1- All isolates which were previously screened for invasion efficiency (Nightingale, unpublished), including their ribotype, lineage and source from which they were isolated (genenv= general environment)

Supplementary Table 2- Alignment of inlA amino acid sequence for isolate containing PMSC 5 (R2-080), isolate containing PMSC 7 (L3-401) and control WT control isolate (EGD-E). Premature stop codon is highlighted for two mutant strains.

isolate	ribotype	lineage	source
fslh4013	116110s2	3	farm
fsln3254	116110s2	3	farm
fsln1211	116110s2	3	human
fslr2311	116239s2	1	human
fsln3768	116239s2	1	farm
fsln3678	116239s2	1	farm
fslf2435	116239s2	1	human
fsln3275	116313s8		farm
fslf2375	116321s3	3	human
fslf2140	116363s2	1	human
fslf2031	116363s2	1	human
fslf2402	116521s6	2	human
fslf2593	116610s3		human
fslt1255	116693s6		foodproc
fsle1158	116741s3		farm
fslr2634	116819s1		human
fsln3337	116849s4		farm
fsln4501	116873s7		farm
fsln4571	116878s6		farm
fsln3167	116890s3		farm
fsln3169	116890s3		farm
fslf3047	116915s2		farm
fsls6016	116931s4		genenv
fsls4154	116931s4		genenv
fslf2090	dup10142	3	human
fslf2086	dup10142	3	human
fslj2076	dup10145	3	food
fslf2208	dup10148	3	human
fslc1418	dup10148	3	human
fslr2304	dup1023a	2	human
fsln4231	dup1023a	2	farm
fsln3163	dup1023a	2	farm
fsls4649	dup1023a	2	genenv
fsle1258	dup1023b	2	farm
fsls4658	dup1023b	2	genenv
fslh4110	dup1023b	2	farm
fslj1054	dup1026	1	human
fsll4336	dup1027a	1	foodproc
fsln3073	dup1027a	1	farm
fslr2245	dup1027a	1	food
fslf2050	dup1027b	1	human
fslr2080	dup1029a	2	food
fslr2081	dup1029a	2	food
fslf2292	dup1029b	2	human
fslf2319	dup1030a	2	human
fslf2453	dup1030a	2	human
fslr2343	dup1030a	2	food
fsln4239	dup1030a	2	farm
fslf2130	dup1030b	2	human
fslh4364	dup1030b	2	farm
fsls4461	dup1030b	2	genenv

fslf2039	dup1030b	2	human
fslf2117	dup1030c	2	human
fslj1039	dup1035	2	human
fslf2448	dup1035a	2	human
fslf2403	dup1035a	2	human
fslr2408	dup1038a	1	food
fsll3175	dup1038a	1	foodproc
fsls6151	dup1038a	1	genenv
fslf2433	dup1038b	1	human
fslh4246	dup1038b	1	farm
fsll4090	dup1038b	1	foodproc
fsls4268	dup1038b	1	genenv
fsln3034	dup1039a	2	farm
fslf2415	dup1039a	2	human
fsls6131	dup1039a	2	genenv
fslr2398	dup1039b	2	food
fslf2225	dup1039b	2	human
fsll4352	dup1039b	2	foodproc
fslt1061	dup1039c	2	foodproc
fslr2169	dup1039c	2	food
fsls4821	dup1039c	2	genenv
fslf2741	dup1039c	2	farm
fslf3049	dup1039d	2	farm
fslf2604	dup1039d	2	human
fsln3155	dup1039e	2	farm
fsls4236	dup1039e	2	genenv
fslc1111	dup1039e	2	human
fsln4015	dup1039e	2	farm
fslr2119	dup1040a	1	food
fslr2200	dup1041a	2	food
fslr2115	dup1041a	2	food
fsls6142	dup1042a	1	genenv
fslf2320	dup1042a	1	human
fslh4012	dup1042a	1	farm
fslr2092	dup1042a	1	food
fsls4848	dup1042b	1	genenv
fslf2051	dup1042b	1	human
fslh4112	dup1042b	1	farm
fsll4025	dup1042b	1	foodproc
fsls6023	dup1042c	1	genenv
fslr2229	dup1042c	1	food
fsll3111	dup1042c	1	foodproc
fslf3228	dup1042c	1	farm
fslf2244	dup1042d	1	human
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fslf2212	dup1043	1	human
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fslr2403	dup1043a	1	food
fsln3277	dup1043a	1	farm
fslr2294	dup1043a	1	human
fsls6134	dup1044a	1	genenv
fsll4408	dup1044a	1	foodproc

fsln3327	dup1044a	1	farm
fslj1213	dup1044a	1	human
fsln3065	dup1044b	1	farm
fsls6093	dup1044b	1	genenv
fsll4241	dup1044b	1	foodproc
fslf2552	dup1044b	1	human
fsls4859	dup1044c	1	genenv
fslf2217	dup1044c	1	human
fslf2038	dup1044d	1	human
fslr2083	dup1044e	1	food
fslr2071	dup1044e	1	food
fslr2127	dup1044e	1	food
fslr2070	dup1044e	1	food
fsls4019	dup1045a	2	genenv
fslf2323	dup1045a	2	food
fsln4046	dup1045a	2	farm
fsle1256	dup1045a	2	farm
fslt1269	dup1045b	2	foodproc
fslr2167	dup1045b	2	food
fsls4295	dup1045b	2	genenv
fsln3480	dup1045b	2	farm
fslf2513	dup1045c	2	human
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fsln4005	dup1045d	2	farm
fsln3064	dup1045d	2	farm
fslc1099	dup1045d	2	human
fsln3175	dup1045e	2	farm
fsln3088	dup1045e	2	farm
fsln3093	dup1045e	2	farm
fsln3115	dup1045e	2	farm
fsls6106	dup1046a	2	genenv
fslf2421	dup1046a	2	human
fsln4530	dup1046a	2	farm
fslj1111	dup1047	2	human
fsln3795	dup1047a	2	farm
fsll3401	dup1048a	2	foodproc
fsln4235	dup1048c	2	farm
fslf3314	dup1048d	2	farm
fslf2579	dup1049b	2	human
fslm1006	dup1051b	1	human
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fsln3794	dup1051c	1	farm
fsln3135	dup1051d	1	farm
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fslr2319	dup1051d	1	food
fslr2178	dup1051d	1	food
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fslj1071	dup1052	1	human
fslf2006	dup1052	1	human
fslj1005	dup1052	1	human
fslf2430	dup1053a	2	human

fsle1154	dup1053a	2	farm
fsll3143	dup1053a	2	foodproc
fslr2134	dup1053a	2	food
fsls4548	dup1053b	2	genenv
fsls4722	dup1053b	2	genenv
fsls4899	dup1053c	2	genenv
fsll3425	dup1053c	2	foodproc
fsls4452	dup1054a	2	genenv
fslm2035	dup1054a	2	human
fslf3317	dup1054a	2	farm
fslh4021	dup1054c	2	farm
fslr2493	dup1056a	2	food
fslr2551	dup1056a	2	food
fsls4470	dup1057b	2	genenv
fslr2310	dup1057b	2	human
fslr2524	dup1057c	2	food
fslr2642	dup1057c	2	human
fsll4174	dup1058b	2	foodproc
fsll4001	dup1058b	2	foodproc
fsls4032	dup1058c	2	genenv
fsle1254	dup1058c	2	farm
fslj1031	dup1059a	3	human
fslf2016	dup1059a	3	human
fsls4839	dup1061a	3	genenv
fslf2458	dup1061a	3	human
fslf2525	dup1061a	3	human
fsln3232	dup1062b	2	farm
fslc1147	dup1062b	2	human
fslr2307	dup1062b	2	food
fsln4463	dup1062c	2	farm
fslf2043	dup1062c	2	human
fslf2434	dup1062c	2	human
fslt1045	dup1062d	2	foodproc
fslr2347	dup1062d	2	food
fslf3217	dup1062d	2	farm
fslf2029	dup1062d	2	human
fsln4500	dup1062e	2	farm
fsln3817	dup1062e	2	farm
fsln3766	dup1062e	2	farm
fsln3880	dup1062e	2	farm
fsln3263	dup1062f	2	farm
fsln3259	dup1062f	2	farm
fsln3344	dup1062f	2	farm
fsls4635	dup16635b	2	genenv
fslf3320	dup16635b	2	farm
fslf2730	dup16635b	2	farm
fslf2270	dup18007a	2	human
fslf2407	dup18036	3	human
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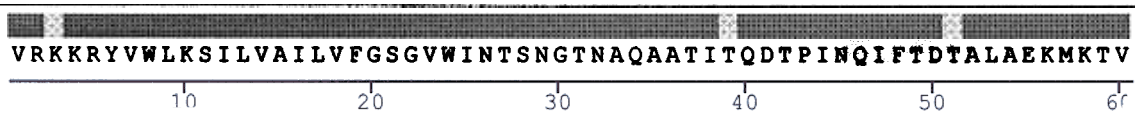


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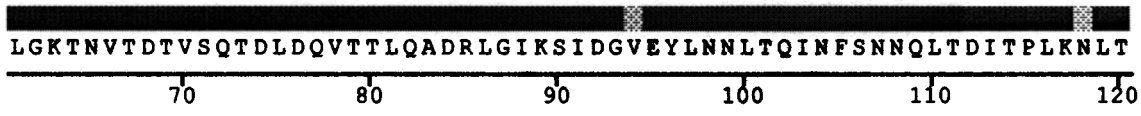


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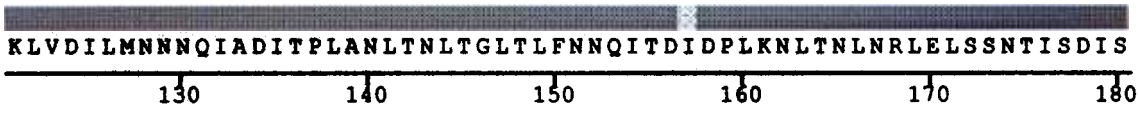


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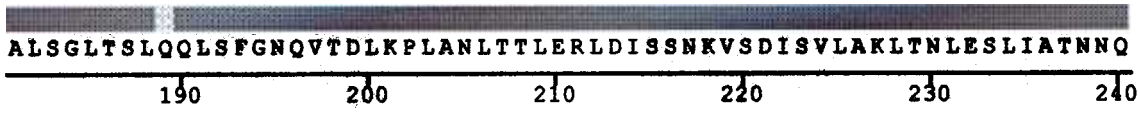


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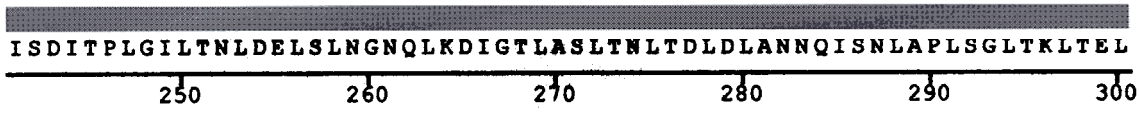


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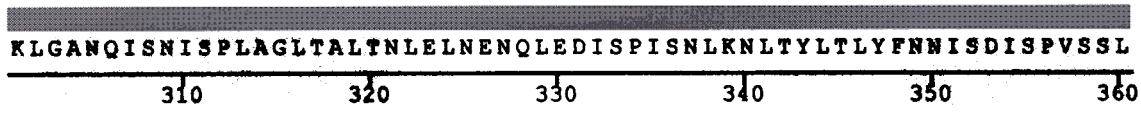


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r2080i lAFULLcutt KLG          ISPLAGLTALTNLELNENQLEDISPISNLKNLTYLTLYFNNISDISPVSSL 1078
l3401i lAFULLcutt KLG          ISPLAGLTALTNLELNENQLEDISPISNLKNLTYLTLYFNNISDISPVSSL 1078
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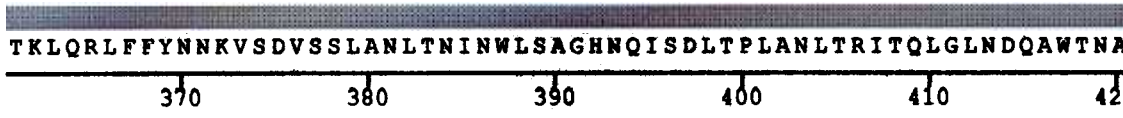


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r2080inlAFULLcutt TKLQRLFFYNNKVSDVSSLANLTNINWLSAGHNQISDLTPLANLTRITQLGLNDQAWTNA 1258
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EGDeFLinlA.seq    TKLQRLFFYNNKVSDVSSLANLTNINWLSAGHNQISDLTPLANLTRITQLGLNDQAWTNA 1258
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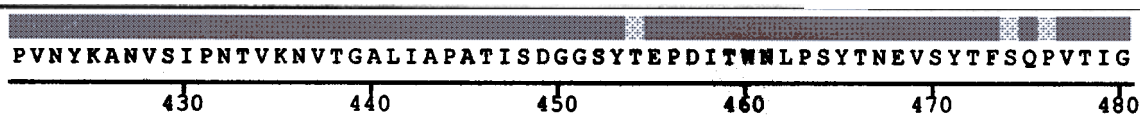


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r2080inlAFULLcutt PVNYKANVSIPNTVKNVTGALIAPATISDGGSYAEPDITWNLPSYTNEVSYTFNQSVTIG 1438
l3401inlAFULLcutt PVNYKANVSIPNTVKNVTGALIAPATISDGGSYTEPDITWNLPSYTNEVSYTFSQPVTIG 1438
EGDeFLinlA.seq    PVNYKANVSIPNTVKNVTGALIAPATISDGGSYTEPDITWNLPSYTNEVSYTFSQPVTIG 1438
```


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r2080inlAFULLcutt KGTTTFSGTVTQPLKAIFNAKFHVDGKETTKEVEAGNLLTEPAKPVKEGYTFIGWFDAKT 1618
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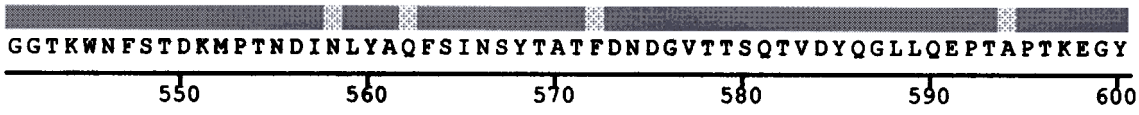


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r2080i lAFULLcutt G  IFSTDKMPTN  DLYAQFSINSYTATLDNDGVTTSQTVDYQGLLQEPTAPTKEGY 1798
l3401i lAFULLcutt G  IFSTDKMPTN  NLYA.FSINSYTATFDNDGVTTSQTVDYQGLLQEPTPPTKEGY 1798
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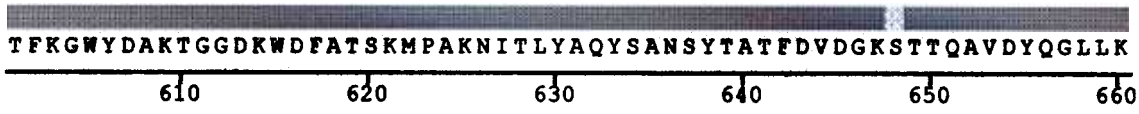


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r2080inlAFULLcutt TFKGWYDAKTGGDKWDFATSKMPAKNITLYAQYSANSYTATFDVDGKTTTQAVDYQGLLK 1978
l3401inlAFULLcutt TFKGWYDAKTGGDKWDFATSKMPAKNITLYAQYSANSYTATFDVDGKSTTQAVDYQGLLK 1978
EGDeFLinlA.seq    TFKGWYDAKTGGDKWDFATSKMPAKNITLYAQYSANSYTATFDVDGKSTTQAVDYQGLLK 1978
```


```
r2080inlAFULLcutt EPKTPTKAGYTFKGWYDEKTDGKKWDFATDKMPANDITLYAQFTKNPVAPPTTGGNTPPT 2158
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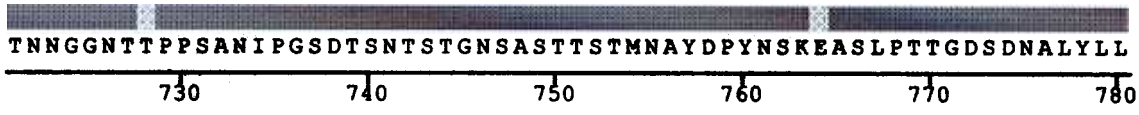


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EGDeFLinlA.seq    TNNGGNTTPPSANIPGSDTSNTSTGNSASTTSTMNAYDPYNSKEASLPTTGDSDNALYLL 2338
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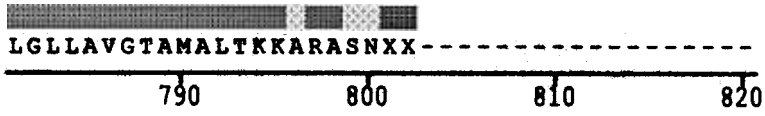


```
r2080inlAFULLcutt LGLLAVGTAMALTKKARARNRR                                  2404
l3401inlAFULLcutt LGLLAVGTAMALTKKHRASNE                                   2401
EGDeFLinlA.seq    LGLLAVGTAMALTKKARASK.K.CKELDVVFGLYLAFLFC                2458
```